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PRINCIPAL INVESTIGATOR: Dr. Daotai Nie

CONTRACTING ORGANIZATION: Southern Illinois University
Springfield, IL 62794

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Dr. Daotai Nie

E-Mail: dnie@siumed.edu

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14. ABSTRACT

Dissemination and metastasis of tumor cells are major causes of morbidity and mortality in breast cancer patients. Therefore it is of vital importance to identify druggable targets to inhibit breast tumor invasion and metastasis. A critical component in the invasive growth, dissemination, and metastasis of cancer is acquisition of motility by tumor cells. Our preliminary studies suggest a novel role for a classical G protein-coupled receptor (GPCR), the thromboxane A2 receptor (TP), in controlling breast tumor cell motility via regulating cytoskeleton reorganization. The objective of this proposal is to define the function of TP in tumor cell motility and to validate TP as a target for anti-metastasis therapy of breast cancer. In the first aim, the role of TP in breast tumor cell motility will be determined in the presence or absence of TP activation or inhibition. The isoform(s) of TP involved in tumor cell contraction and motility will be identified in the second specific aim. In the third aim, an orthotopic mouse model will be used to assess whether TP can be targeted to reduce breast cancer metastasis. The proposed studies will provide significant insights into how the tumor cells enlist TP GPCR to regulate cell motility, and will be highly significant toward the goals of developing mechanism-based interventions for cancers.

15. SUBJECT TERMS
Breast cancer, metastasis, thromboxane, G protein coupled receptor, migration, invasion

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Introduction

Major causes of death in breast cancer patients are the spread and metastasis of tumors. "Metastasis" means that the cancer cells have detached from the original tumor site and started to grow in another part of the body. The goal of this research project is to find out how breast cancer cells disseminate and move so that new methods can be developed to identify and treat breast cancer. For a tumor cell to move, it must push forward in the front and contract in the rear to power ahead. The main idea of the proposed project is that tumor dissemination and migration can be inhibited by blocking tumor cell contraction. Our laboratory has identified a cell receptor (TP) that receives information ("receptor") in breast tumors and then participates in cell contraction and migration. This receptor can be activated by a lipid called thomboxane A_2. A published study suggests that the level of this receptor expressed in breast tumor tissues have been linked with poor prognosis and a significant decrease in disease free survival. Our preliminary studies suggest that when thromboxane A_2 receptor is activated, breast cancer cells immediately contract. If the activation of this receptor is blocked, breast cancer cells cannot move and spread. We hypothesize that TP (TxA_2 receptor) regulates the motility of carcinoma cells by elaborating the reorganization of cytoskeleton during migration and that inhibition of TP activation can reduce the motility, invasion, and metastasis of breast carcinoma cells. The objective of this proposal is to define the function of TP in tumor cell motility and to validate TP as a target for anti-metastasis therapy of breast cancer with the following aims:

Aim 1. Define the role of TP activation in breast cancer cell motility.

Aim 2. Determine the isoform(s) of TP involved in cytoskeleton reorganization in motility of tumor cells.

Aim 3. Validate TP(s) as a target for treatment of breast cancer metastasis.

BODY OF REPORT

Task 1. Define the role of TP activation in breast cancer cell motility (Months 1 – 12).

Most studies in this task have been achieved in the first year of funding, and reported in the first annual report.

Task 2. Determine the isoform(s) of TP involved in cytoskeleton reorganization in motility of tumor cells (Months 13-24)

Human thromboxane A_2 receptor (TP) is expressed as two different isoforms, TPα and TPβ, that arise by alternative mRNA splicing. To examine the expression of TPs in human breast carcinoma cells, we used isoform-specific PCR primer sets to amplify a 268-bp fragment of TPα and a 330-bp fragment of TPβ. We examined the expression of TPalpha and TPbeta in a number of breast cancer cell lines and found that while TPalpha is expressed in all cell lines examined, TPbeta is expressed in more malignant cells such as MDA-MB-231 cells, and Rao3 and Rao 4 cells, which are H-Ras transformed, highly malignant triple negative breast cancer cells.

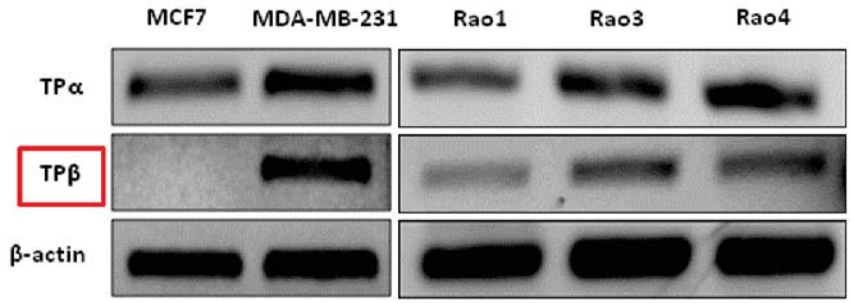

Figure 1. Expression of TPα and TPβ in breast carcinoma cancer cells as revealed by RT-PCR using isoform specific primers.

Interestingly, treatment of MDA-MB-231 cells with CAY10535, a TP antagonist that has 20 fold selectivity for TPβ relative to TPα was enough to block U46619 induced contraction as shown in the **Figure 2D** in the last report and for simplicity, also appended below. The data suggest that contraction process in MDA-MB-231 cells may be functioned through the activation of TPβ.

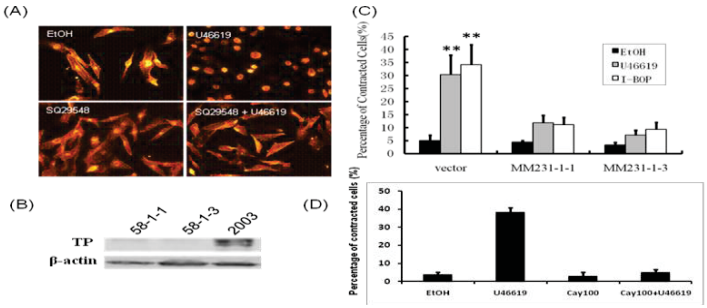

Figure 2. A. Induction of cell contraction by TP agonist, U46619, and the inhibition of cell contraction by TP antagonist, SQ29548. B. Generation of stable transfectants with TP expression ablated using shRNA constructs in MDA-MB-231 cells. 58-1-1, 58-1-3: shRNA constructs, 2003: vector control. C. Blockade of U46619-induced cell contraction by down regulation of TP in MDA-MB-231 cells. 1-1 and 1-3 are two stable transfectants with TP expression ablated. **, P<0.01 when compared with vector control. D. Blockade of U46619-induced cell contraction by CAY10535, antagonist of TPβ. Cells were pretreated with CAY10535 at 100nM for 15 min before the treatment with 200 nM U46619. ***, P < 0.001, when compared with its vehicle control [ethanol (EtOH)].

To detect TPbeta expression in breast cancers, we used multi pronged approaches. First, we have generated rabbit polyclonal antibody against TPbeta specific peptide sequences. Currently we are validating the specificity of the polyclonal antibody.

The antibody developed was not working well as expected.

Second, we initiated collaboration with Dr. Kinsella in Ireland, who has TPalpha and TPbeta specific antibodies. Using these isoform specific antibodies, we performed IHC analysis of TP expression in breast cancer tissues and analyzed their correlations with clinical and pathological parameters.

We found that: 1) TPbeta expression is significantly increased in breast cancer patients with distant metastasis when compared with metastasis free patients (**Figure 3**).

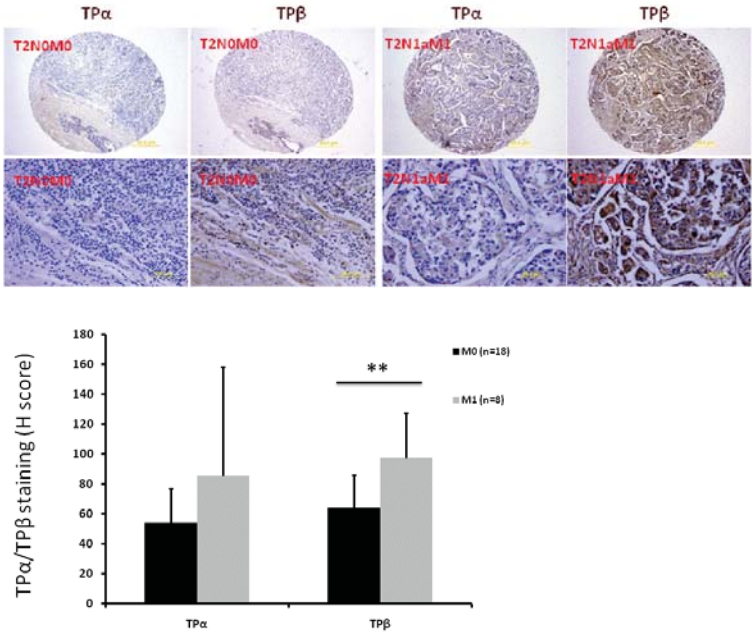

Figure 3. Expression levels of TPα/TPβ in human breast tissues array categorized according to TNM grading. M0 – no distant metastasis, M1 – distant metastasis. **, p < 0.01 when compared with M0 group.

2) TPbeta expression is significantly increased in breast cancer patients with larger tumor size (> 3cm) compared with those have smaller tumor size (< 3cm), and in tumors with high grades (**Figure 4**).

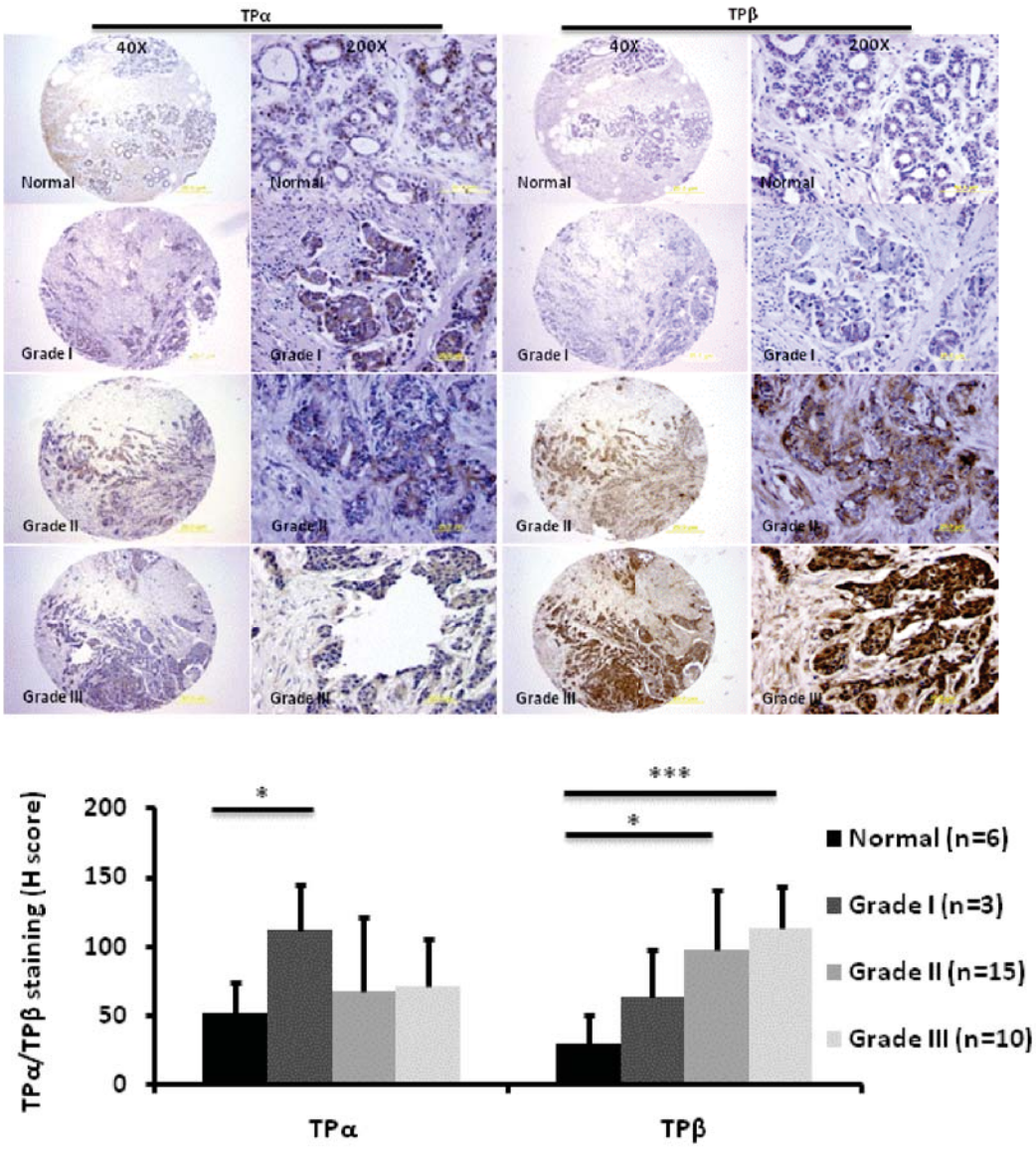

Figure 4. Expression of TPalpha and TPbeta in breast tumors of different grades. Note the increased expression of TPbeta in high grade tumors.

3) Neither TPα nor TPβ expression level correlates with patients' age/ clinical outcome/ No. of positive lymph nodes/ ER/ PR/ p53 status/ tumor grade, according to the specimens we analyzed so far.

Third, we are continuing to purify TPbeta specific recombinant protein to generate TPbeta specific antibody for future diagnosis.

Task 3. Validate TP as a target for treatment of breast cancer. (Months 6 – 36). This objective will be started in the first year of funding and kept on continuous basis until the end of three year period.

Acquisition of motility by breast cancer cells is a key component in metastasis. To evaluate whether ablation of TP expression in MDA-MB-231 cells will result in altered metastatic potentials *in vivo*, we have generated several sublines of MDA-MB-231 cells with TP stably knocked down (**Figure 2**), and further labeled them with the firefly luciferase gene using VSV-g pseudotyped Lentiviral vector, MDA-MB-231-2003-Luc, MDA-MB-231-231-58-1-1-Luc and MDA-MB-231-231-58-1-3-Luc cells.

To study which step is blocked by TP knock down in MDA-MB-231 in the metastatic process, we did soft agar assay to check out the anchorage-independent ability. As shown in **Figure 5**, down regulation of TP in MDA-MB-231 cells did not affect its anchorage-independent growth ability.

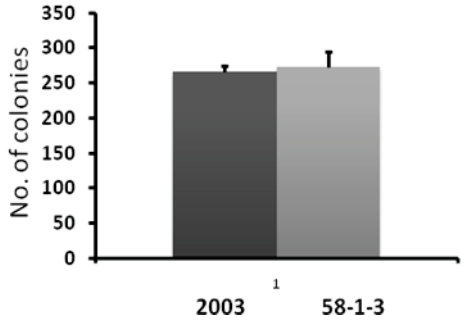

Figure 5. Anchorage-independent growth of MDA-MB-231 cells, the cells were resuspended in medium supplemented with agar at a final concentration of 0.3%, and layered with RPMI1640 supplemented with 0.6% agar, then cultured for 14 days.

When implanted in the mammary fat pad of female nude mice, the down regulation of TP in MDA-MB-231 cells did not affect the growth of the primary tumors significantly when compared with control tumors (data not shown). However, since we have to sacrifice the mice when the sizes of primary tumors reach 2 cm^3, at which time point, we did not detect metastatic lesions in the lung or liver. Currently we are developing imaging methods to detect metastasis from primary tumors.

Next, MM231-58-1-1 and -58-1-3 with TP stably knocked down, as well as vector control cell line MM231-2003 were injected into SCID mice via tail vein. Those mice injected with TP knockdown MDA-MB-231 cells presented fewer lung metastasis nodules than those injected with vector control cells (**Figure 6A, B)**. Moreover, by measuring luciferase activity in the organs harvested from the mice as an indicator for metastatic burdens, we found a significant reduction in luciferase readout in the livers from the mice injected with TP knockdown MDA-MB-231 cells as compared to those injected with control cells (**Figure 6C**). It should be noted

that in a spontaneous metastasis model, TP ablation in MDA-MB-231 cells did not affect either the primary tumor formation or the primary tumor growth rate. These data suggest that TP-mediated signaling is required for tumor metastasis.

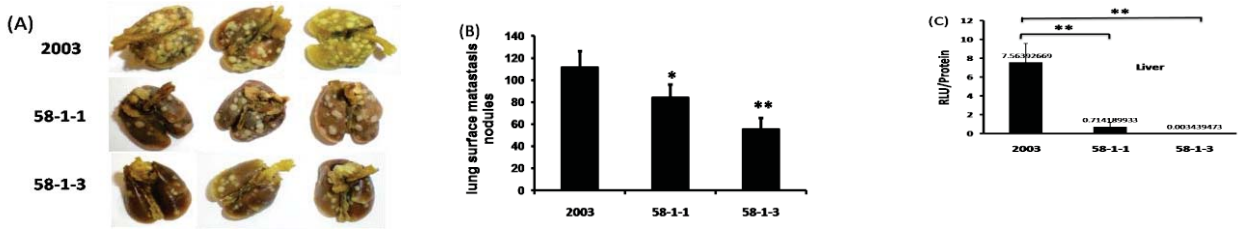

Figure 6. Inhibition of development of experimental metastasis by TP ablation (A) Gross morphology of resected mouse lungs 30 days after tail vein injection of MDA-MB-231-2003 (vector control), MDA-MB-231-58-1-1 and MDA-MB-231-58-1-3 (TP knockdown) cells. (B) Reduction of metastatic lesions by TP ablation. Columns, average number of metastatic lesions per mouse. (C) Metastasis to the liver as indicated by the normalized luciferase readout.

KEY RESEARCH ACCOMPLISHMENT and REPORTABLE OUTCOMES

Presentations:

Xuejing Zhang, Man-Tzu Wang, Yong Tang, Yakun Chen, and **Daotai Nie**. Thromboxane A2 receptor (TP) in tumor cell motility and metastasis. 100th AACR Annual Meeting, Denver, CO. April 18-22, 2009.

Daotai Nie. Nanog, cancer stem cells, and a switch in breast cancer metastasis. Invited presentation. University of Kansas Cancer Center. Kansas City, KS. March, 2010.

Daotai Nie. Cancer stem cells and a switch in metastasis. Invited presentation. University of South Florida College of Medicine and Moffit Cancer Center, Tampa, FL. April, 2010.

Zhang Xuejing, Wang Man-Tzu, Chen Yakun, Yong Tang and Nie Daotai. Regulation of breast cancer metastasis by thromboxane A2 receptor signaling. Joint Metastasis Research Society-AACR Conference for Metastasis and the Tumor Microenvironment, Philadelphia, PA. September 12 -15, 2010.

Abstracts published:

Xuejing Zhang, Man-Tzu Wang, Yong Tang, Yakun Chen, and **Daotai Nie**. Thromboxane A2 receptor (TP) in tumor cell motility and metastasis. Proc. Amer. Assoc. Cancer Res. 48: #3098, 2009.

Grants submitted:

Xuejing Zhang (PI), Daotai Nie (mentor). Aspirin, Thromboxane, and Breast Cancer Metastasis. Department of Defense Breast Cancer Research Program Predoctoral Training Grants.

Articles published:

Tang Y, and D. Nie. Role of GTPases in tumor cell migration and metastasis. Cell Movement (F Columbus, ed.). Nova Science Publisher, Hauppauge, NY. 2009.

Chen Y, Tang Y, Chen S, and Nie D. Regulation of breast cancer cell responses to chemotherapy by pregnane X receptor. Cancer Biology & Therapy 8: 1265 - 1272. 2009.

Walia V, Ming D, Kumar S, Nie D, and Elble R. hCLCA2 is a p-53-inducible inhibitor of breast cancer cell proliferation. Cancer Research 69: 6624 – 6632, 2009. PMID: 19654313.

Zhang X, Nie D, and Chakrabarty S. Growth factors in tumor microenvironment. Frontiers in Biosciences 15: 151 - 165, 2010.

CONCLUSIONS:

The receptor of thromboxane A_2 (TXA_2), TP, was found to correlate with a poor prognosis in breast cancer patients, as suggested by the inverse correlation between TP mRNAs and disease free survival of patients. Our studies here provide mechanistic insights into how TP is involved in breast cancer progression especially metastasis.

We found TP is expressed at protein levels and further TP is functional. Our studies identify TPbeta isoform as the receptor increasingly expressed in high grade tumors, especially in tumors with distant metastasis. TPbeta expression was found selectively limited to breast cancer cells of high malignancy as well.

Treatment of human breast cancer cell lines MDA-MB-231 by U46619, a TP agonist, induced cell body contraction which can be blocked by a TPβ specific inhibitor CAY10535. The results suggest a selective role of TPbeta in cell contraction.

Third, TP depletion reduced metastatic potential of breast cancer cells. MDA-MB-231 cells with TP knockdown displayed a reduced percentage of contraction under the treatment of U46619 and TXA_2 agonist I-BOP as well as attenuated invasion ability. The depletion of TP affected neither the proliferation, nor the anchorage independent growth ability of the cells. After injection into mice via the tail vein, TP depletion reduced the ability of MDA-MB-231 cells to metastasize to the lung and the liver. The results indicate that depletion of TP reduces the metastatic dissemination of breast cancer cells *in vivo.*

Further studies are ongoing to develop strategy to block TP activation in vivo to reduce breast cancer metastasis.

So what? Our studies are significant in the following ways: 1) TP, especially TPbeta, can be a promising target to develop treatment to block breast cancer metastasis. 2) Inhibition of thromboxane A2 production, either using TX synthase inhibitor or aspirin or other cyclooxygenase inhibitors may reduce breast cancer metastasis. This exciting possibility, as proposed in our predoctoral training grant application, needs further studies.

REFERENCES

N/A

APPENDICES

N/A

SUPPORTING DATA

Embedded in the reporting body